Diplopyrone, a New Phytotoxic Tetrahydropyranpyran-2-one Produced by *Diplodia mutila,* a Fungus Pathogen of Cork Oak

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A new phytotoxic monosubstituted tetrahydropyranpyran-2-one, named diplopyrone (**1**), was isolated from the liquid culture filtrates of *Diplodia mutila*, a plant pathogenic fungus causing a form of canker disease of cork oak (*Quercus suber*). Diplopyrone was characterized, using spectroscopic and chemical methods, as 6-[(1*S*)-1-hydroxyethyl]-2,4a,6,8a-tetrahydropyran[3,2-*b*]pyran-2-one. The absolute stereochemistry of the chiral secondary hydroxylated carbon (C-9), determined by application of Mosher's method, proved to be *S*. Diplopyrone assayed at a 0.01−0.1 mg/mL concentration range caused necrosis and wilting on cork oak cuttings. On a nonhost plant, tomato, diplopyrone caused brown discoloration or stewing on the stem.

Diplodia mutila (Fr.) apud Mont., anamorph of *Botryosphaeria stevensii* Shoem., is an endophytic fungus, widespread in Sardinian oak forests and considered one of the main causes of cork oak (*Quercus suber* L.) decline.[1] The fungus can affect plants of different age, inducing symptoms very similar to those produced by tracheomycotic disease. When inoculated on stems of young cork oak plants, *D. mutila* induced a slight collapse and dark brown discoloration of the cortical tissues around the inoculation site, a sudden wilting of the plant above it, and subsequently a sprouting of secondary shoots below it.[2] These symptoms suggested that the fungus produced phytotoxic metabolites, as also observed for isolates of *D. mutila* from cypress and other oak species.[3] This paper describes the isolation and the chemical and biological characterization of the main phytotoxic metabolite produced by *D. mutila*.

The organic extract obtained from culture filtrates of *D. mutila* was purified using the method described in the Experimental Section. The main toxin **1** (4.5 mg/L), called diplopyrone, was obtained in the form of a homogeneous oil resistant to crystallization. Diplopyrone, assayed at concentrations ranging from 0.01 to 0.1 mg mL^{-1}, was toxic to *Q. suber*. Necrotic lesions appeared on the leaves within 4 days after absorption of the toxic solutions (0.1−0.01 mg mL^{-1}). Cork oak cuttings wilted within 8 days. When **1** was assayed on tomato cuttings, phytotoxicity was evident at 0.2 and 0.1 mg mL^{-1}, inducing internal tissue collapse on the stem. No phytotoxicity was detected at 0.05 and 0.02 mg mL^{-1}.

Compound **1** had a molecular weight of 196, corresponding to a molecular formula of $C_{10}H_{12}O_4$, consistent with the five unsaturations. Absorption bands typical of α,*β*-unsaturated carbonyl groups and hydroxy groups were observed in the IR spectrum.

Preliminary NMR spectra showed three out of five of unsaturations were consistent with an α,*β*-unsaturated ester carbonyl group. Analysis of the ^{1}H and ^{13}C NMR spectra (Table 1) confirmed these structural features. The

Table 1. ^{1}H and ^{13}C NMR Spectral Data of Diplopyrone (**1**)[a,b]

C	δ[c]	^{1}Hδ	*J*(Hz)	HMBC
2	162.7 (s)			6.88, 6.23
3	124.8 (d)	6.23 (d)	(9.8)	4.09
4	140.0 (d)	6.88 (dd)	(9.8, 5.8)	4.09
4a	64.9 (d)	4.09 (dd)	(5.8, 2.8)	6.88, 6.23
6	78.9 (d)	4.16 (br s)	(4.2, 3.5, 1.8)	6.18, 6.14, 1.21
7	132.6 (d)	6.18 (d)	(10.4)	4.65
8	123.0 (d)	6.14 (ddd)	(10.4, 4.6, 1.8)	4.65
8a	69.7 (d)	4.65 (ddd)	(4.6, 3.5, 2.8)	6.88, 4.09
9	69.03 (d)	3.92 (dq)	(6.5, 4.2)	6.18, 6.14, 1.21
10	17.8 (q)	1.21 (3H, d)	(6.5)	

[a] The chemical shifts are in δ values (ppm) from TMS. [b] 2D ^{1}H,^{1}H (COSY, TOCSY) and 2D ^{13}C,^{1}H (HSQC) NMR experiments delineated the correlations of all protons and the corresponding carbons. [c] Multiplicities determined by DEPT spectrum.

^{1}H NMR spectrum showed a double doublet (J = 9.8 and 5.8 Hz) and a doublet (J = 9.8 Hz) at δ 6.88 and 6.23 due to H-4 and H-3, respectively, of a *cis*-disubstituted olefinic group conjugated to a carbonyl group.[4,5] The latter had an ester nature as deduced from the chemical shift of the corresponding singlet appearing at δ 162.7 in the ^{13}C NMR spectrum (Table 1).[6] The two olefinic protons correlated in the HSQC spectrum[7] with the corresponding carbons recorded at the expected chemical shift values of δ 140.0 (C-4) and 124.8 (C-3).[6] The H-4, in the COSY and TOCSY spectra,[7] coupled with the proton of an adjacent secondary oxygenated carbon (C-4a), resonating as a double doublet (J = 5.8 and 2.8 Hz) at δ 4.09. This in turn coupled with the proton of another secondary oxygenated carbon (C-8a), which appeared as a doublet of double doublets (J = 4.6, 3.5 and 2.8 Hz) at δ 4.65. The latter, which in the HSQC spectrum correlated with a carbon appearing at the typical shift value of δ 69.7, represents the closure point of the δ-lactone ring (a 5,6-dihydro-2*H*-pyran-2-one) present in **1**. Furthermore, H-8a coupled in the COSY and TOCSY spectra with the adjacent proton (H-8) of another *cis*-disubstituted double bond and also with H-6 by a typical homoallylic coupling constant (J = 3.5 Hz).[5] H-8 appeared at δ 6.14 as a doublet of double doublets (J = 10.4, 4.6, and 1.8 Hz), being coupled with the other olefinic proton H-7, a doublet (J = 10.4 Hz) resonating at δ 6.18, with H-8a as described above, and with H-6 by a typical allylic

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coupling (J = 1.8 Hz).[4,5] The latter is the carbinol proton of another secondary oxygenated carbon (C-6) and was observed at δ 4.16. It appeared as a complex signal (J = 4.2, 3.5, and 1.8 Hz) because of the coupling with H-8 and H-8a, but also for its further coupling with the proton of a secondary hydroxylated carbon (C-9) of a 1-hydroxyethyl side chain. H-9 resonated as a double quartet (J = 6.5 and 4.2 Hz) at δ 3.92, as it was also coupled with a terminal methyl group (Me-10), which was observed as a doublet (J = 6.5 Hz) at δ 1.21.[4] The molecular formula of **1** presents a total of five unsaturations, three of which are included into the α,*β*-unsaturated *δ*-lactone and one due to the additional double bond. Of the four oxygen atoms, two are included in the lactone ring and one in the hydroxy group of the 1-hydroxyethyl side chain at C-6. The remaining oxygen atom and unsaturation are consistent with a 3,6-dihydro-2*H*-pyran ring fused to that of the 5,6-dihydro-2*H*-pyran-2-one. The olefinic protons (H-7 and H-8) and H-6 of the 3,6-dihydro-2*H*-pyran ring, those of the bridgehead carbons (H-4a and H-8a), and those of the secondary hydroxylated carbon and methyl group of the 1-hydroxyethyl side chain correlated in the HSQC spectrum with the corresponding carbon appearing in the ^{13}C NMR spectrum (Table 1) at the expected chemical shift values at δ 132.6, 123.0, and 78.9 (C-7, C-8 and C-6), δ 69.7 and 64.9 (C-8a and C-4a), and δ 69.03 and 17.8 (C-9 and C-10).[6]

The presence of the above structural features in **1** was confirmed by the correlation observed in the COSY, TOCSY, and HSQC spectrum, which also allowed us to assign, in agreement with the literature,[4,6] the chemical shifts to all protons and carbons (Table 1) and to suggest for the toxin the structure of 6-(1-hydroxyethyl)-2,4a,6,8a-tetrahydropyran[3,2-*b*]pyran-2-one (**1**).

This structure was supported by the ^{1}H,^{13}C long-range correlations recorded for **1** in the HMBC spectrum (Table 1)[7] and by data of its EIMS spectrum. The latter, in addition to the molecular ion at 196.0726, showed the corresponding protonated ion $[M + H]^+$ at *m/z* 197, frequently observed for lactone ring containing compounds.[4,8] These two ions, by alternative and successive losses of H_2O and CO_2 molecules, generated two series of ions at *m/z* 179, 153, and 135 and 178, 152, and 134, respectively.[4,8] The ions at *m/z* 178 and 153, by alternative losses of Me and CH_3CHO residues, produced the ions at *m/z* 163 and 109, respectively.[4,8] The ESMS spectrum showed the potassium $[M + K]^+$ and the sodium $[M + Na]^+$ clusters and the pseudomolecular ions at respectively *m/z* 235, 219, and 197.

The stereochemistry of the bicyclic moiety of **1** was deduced from the $J^3_{H,H}$ coupling constants. In fact, a *cis*-configuration for the junction between the two dihydro-2*H*-pyran rings, both of which probably adopt a half-chair conformation, was deduced by comparing the coupling value between H-4a and H-8a (J = 2.8 Hz) with those reported for model compounds.[4,5] The lack of coupling between H-7 and H-6 located the latter proton in the axial position and consequently the 1-hydroxyethyl group equatorially. Therefore, in agreement with the NOESY data[7] (Table 2) and after inspection of a Dreiding model of **1**, a relative stereochemistry with the bridgehead hydrogens (H-4a and H-8a) on the same side of the molecule and opposite to H-6 is suggested for diplopyrone. The stereochemistry of the secondary hydroxylated carbon of the 1-hydroxyethyl side chain at C-6 was determined applying the Mosher's method.[9,10] Diplopyrone, by reaction with the *R*-(−)-α-methoxy-α-trifluorophenylacetate (MTPA) and *S*-(+)MTPA chlorides, was converted to the corresponding diastereomeric *S*-MTPA (**2**) and *R*-MTPA (**3**) esters, whose spectroscopic data were consistent with the structure assigned to **1**. In particular, comparison between the ^{1}H NMR data (Table 3) of the *R*-MTPA ester (**3**) and those of the *S*-MTPA ester (**2**) showed a downfield shift ($\Delta\delta$ 0.09) of Me-10, along with an upfield shift ($\Delta\delta$ 0.10) of H-6. These results, in agreement with literature data,[9,10] allowed the assignment of an *S*-configuration at C-9. Therefore diplopyrone (**1**) can be formulated as 6-[(1*S*)-1-hydroxyethyl]-2,4a,6,8a-tetrahydropyran[3,2-*b*]pyran-2-one.

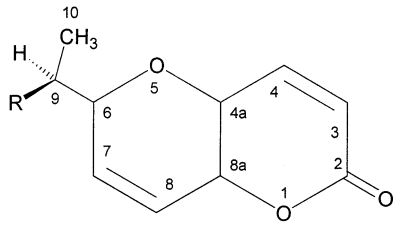
Table 2. 2D ^{1}H NOE (NOESY) Spectral Data Obtained for Diplopyrone (**1**)

considered	effects
6.88 (H-4)	6.23 (H-3), 4.09 (H-4a)
6.23 (H-3)	6.88 (H-4), 4.09 (H-4a)
6.18 (H-7)	4.65 (H-8a), 4.16 (H-6)
6.14 (H-8)	4.65 (H-8a)
4.65 (H-8a)	6.18 (H-7), 6.14 (H-8), 4.09 (H-4a)
4.16 (H-6)	6.18 (H-7), 3.92 (H-9)
4.09 (H-4a)	6.88 (H-4), 6.23 (H-3), 4.65 (H-8a)
3.92 (H-9)	4.16 (H-6), 1.21 (Me-10)
1.21 (Me-10)	3.92 (H-9)

Table 3. ^{1}H NMR Data of the (*S*)- and (*R*)-α-Methoxy-α-trifluorophenylacetate (MTPA) Esters of Diplopyrone (**2** and **3**)[a]

	2		**3**	
H	*δ*	*J* (Hz)	*δ*	*J* (Hz)
3	6.19 (d)	(9.8)	6.24 (d)	(9.8)
4	6.78 (dd)	(9.8, 5.8)	6.85 (dd)	(9.8, 5.8)
4a	4.00 (dd)	(5.8, 2.8)	4.07 (dd)	(5.8, 2.8)
6	4.23 (br s)	(4.9, 3.6, 1.6)	4.33 (br s)	(3.7, 2.5, 1.9)
7	5.97 (d)	(10.3)	6.10 (d)	(10.5)
8	6.10 (ddd)	(10.3, 5.6, 1.6)	6.96 (ddd)	(10.5, 5.1, 1.9)
8a	4.60 (ddd)	(5.6, 3.6, 2.8)	4.65 (ddd)	(5.1, 2.8, 2.5)
9	5.18 (dq)	(6.4, 4.9)	5.27 (dq)	(6.6, 3.7)
10	1.38 (3H, d)	(6.4)	1.29 (3H, d)	(6.6)
OCH_3	3.52 (s)		3.53 (s)	
Ph	7.52−7.42 (m)		7.51−7.39 (m)	

[a] The chemical shifts are in *δ* values (ppm) from TMS.



1 R=OH

2 R=*S*-MTPA

3 R=*R*-MTPA

Pyran-2-ones (α-pyrones) are a group of naturally occurring compounds that are broadly distributed in nature as plant, animal, marine organism, and microbial metabolites, most with interesting biological activity,[11−13] and the total synthesis of some of them has been achieved.[13] Other secondary metabolites containing the pyran-2-one moiety are produced by fungi belonging to several genera including *Alternaria*, *Aspergillus*, *Fusarium*, and *Trichoderma* and exhibit antibiotic, antifungal, cytotoxic, neurotoxic, and phytotoxic activities.[14]

Experimental Section

General Experimental Procedures. Optical rotation was measured in $CHCl_3$ on a JASCO DIP-370 digital polarimeter;

IR and UV spectra were determined as neat and in MeOH solution, respectively, on a Bio-Rad Win FT-IR spectrometer and a Perkin-Elmer Lambda 3B spectrophotometer; ^{1}H and ^{13}C NMR spectra were recorded at 500, 400, or 300 MHz and at 125, 100, or 75 MHz, respectively, in $CDCl_3$, on Bruker spectrometers. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectrum.[7] DEPT, COSY-45, TOCSY, HSQC, HMBC, and NOESY experiments[7] were performed using Bruker microprograms. EI and HREIMS were taken at 70 eV and on a Fisons Trio-2000 and a Fisons ProSpec spectrometer, respectively. Electrospray MS were recorded on a Perkin-Elmer API 100 LC-MS; a probe voltage of 5300 V and a declustering potential of 50 V were used. Analytical and preparative TLC were performed on silica gel (Merck, Kieselgel 60, F_{254}, 0.25 and 0.5 mm, respectively) or on reversed-phase (Merck, RP-18, F_{254}, 0.25 mm) plates. The spots were visualized by exposure to UV radiation and/or by dipping the plates in a 10% (w/v) aqueous solution of $KMnO_4$ or by spraying with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in MeOH, followed by heating at 110 °C for 10 min. Column chromatography was performed on silica gel (Merck, Kieselgel 60, 0.063−0.20 mm).

Fungal Strain. The *D. mutila* strain used in this study was isolated from stems of infected cork oak (*Q. suber*) trees collected in Sardinia (Italy). A single spore isolate of *D. mutila* was grown on potato-dextrose-agar slants at 25 °C for 10 days and then stored at 5 °C in the fungal collection of the Dipartimento di Protezione delle Piante, Università di Sassari, Italy (PVS 114S).

Production, Extraction, and Purification of 1. The isolate PVS 114S of *D. mutila* was grown in stationary culture in 1 L Roux flasks containing 150 mL of Czapek medium with the addition of 0.5% yeast extract (pH 5.9). The flasks were incubated at 25 °C for 30 days in the dark. At harvest, the mycelium mat was removed by filtration. The culture filtrate (10 L; pH 7.5−8.1) was acidified to pH 4 with 2 N HCl and extracted with EtOAc (5 × 2 L). The combined organic extracts were dried (Na_2SO_4) and evaporated under reduced pressure to give a red-brown oil residue with high phytotoxic activity. The crude residue was fractionated by column chromatography eluted with a gradient of CH_2Cl_2−MeOH (from 50:1 to 1:2, v/v). Fractions (15 mL each) were collected and pooled on the basis of their TLC profiles to yield 10 major fractions (1−10). Only the residue left from fractions 6, 7, and 8 showed high phytotoxic activity. Phytotoxic fraction 6 (558 mg) was applied to a silica gel column, which was eluted with a gradient of $CHCl_3$−*i*-PrOH (from 10:1 to 1:2). Six fractions were obtained. The phytotoxic activity was concentrated in fractions 2 and 3. Further purification of these fractions by successive preparative TLC, eluent EtOAc, yielded **1** (45 mg, 4.5 mg/mL) as a homogeneous oil resistant to crystallization [R_f 0.54, 0.19, and 0.76, by silica gel and reversed-phase TLC, eluent systems $CHCl_3$−*i*-PrOH (9:1), petroleum ether−Me_2CO (7:3), and EtOH−H_2O (1:1), respectively].

Diplopyrone (1): colorless oil; $[\alpha]^{25}_D$ +67.6° (*c* 0.25); UV λ_{max} (log ϵ) 202 (4.37) nm; IR ν_{max} 3436, 1722, 1637, 1258 cm^{-1}; ^{1}H and ^{13}C NMR, see Tables 1 and 2, respectively; HREIMS *m/z* (rel int) 197 $[M + H]^+$ (12), 196.0726 $[M]^+$ (calcd for $C_{10}H_{12}O_4$, 196.0736) (2), 181 $[M - Me]^+$ (4), 179 $[M + H - H_2O]^+$ (12), 178 $[M - H_2O]^+$ (1), 163 $[M - H_2O - Me]^+$ (5), 153 $[M + H - CO_2]^+$ (56), 152 $[M - CO_2]^+$ (98), 135 $[M + H - H_2O - CO_2]^+$ (41), 134 $[M - H_2O - CO_2]^+$ (98), 109 $[M + H - CO_2 - CH_3CHO]^+$ (33), 45 $[C_2H_5O]^+$ (100); ESMS (+) *m/z* 235 $[M + K]^+$, 219 $[M + Na]^+$, 197 $[M + H]^+$.

(*S*)-α-Methoxy-α-trifluorophenylacetate (MTPA) Ester of Diplopyrone (2). (*R*)-(−)-MPTA-Cl (20 μL) was added to diplopyrone (**1**, 2.8 mg), dissolved in dry pyridine (200 μL). The mixture was allowed to stand at room temperature. After 1 h, the reaction was complete, and MeOH was added. The pyridine was removed by a N_2 stream. The residue was purified by preparative TLC on silica gel (petroleum ether−Me_2CO, 7:3), yielding **2** as an oil (3.6 mg): $[\alpha]^{25}_D$ +35.5° (*c* 0.36); UV λ_{max} log(ϵ) 260 (2.76), 205 (4.54) nm; IR ν_{max} 1753, 1722, 1637, 1251, 1181 cm^{-1}; ^{1}H NMR, see Table 3; EIMS *m/z* (rel int) 413 $[M + H]^+$ (3.6), 412 $[M]^+$ (2.4), 397 $[M - CH_3]^+$ (1.4), 393 $[M - F]^+$ (2), 368 $[M - CO_2]^+$ (1.7), 362 $[M - F - CH_3O]^+$ (8), 353 $[M - CO_2 - CH_3]^+$ (0.4), 189 $[C_9H_8OF_3]^+$ (100), 69 $[CF_3]^+$ (67).

(*R*)-α-Methoxy-α-trifluorophenylacetate (MTPA) Ester of Diplopyrone (3). (*S*)-(+)-MPTA-Cl (20 μL) was added to diplopyrone (2.4 mg), dissolved in dry pyridine (200 μL). The reaction was carried out under the same conditions used for preparing **2** from **1**. Purification of the crude residue by preparative TLC on silica gel (petroleum ether−Me_2CO, 7:3) yielded **3** as an oil (3.3 mg): $[\alpha]^{25}_D$ +77.9° (*c* 0.33); UV, IR, and EIMS were very similar to those of **2**; ^{1}H NMR, see Table 3.

Toxin Bioassays. The culture filtrates, their organic extracts, the chromatographic fractions, and pure **1** were assayed for phytotoxicity using cuttings of cork oak and tomato seedlings. The cuttings were taken from cork oak and tomato seedlings (35 and 21 days old, respectively) grown in a growth chamber at 25 °C and 70−80% RH, exposed to a luminous flux of 400 mmol m^{-2} s^{-1} with a 12 h photoperiod. Aliquots of the culture filtrate were assayed after 1:1000 dilution with distilled H_2O. The crude extracts from culture filtrate and residues of column chromatography fractions were assayed at concentrations of 0.1, 0.2, and 0.5 mg mL^{-1}. The pure **1** was tested at concentrations of 10−100 μg mL^{-1} on cork oak cuttings and 20−200 μg mL^{-1} on tomato cuttings. The toxicity of these solutions was evaluated by placing the test plant parts (cork oak cuttings for 96 h and tomato for 48 h) in the assay solutions (3 mL) and then transferring them to distilled H_2O.

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